AAC Journals.ASM.org

Saccharomyces cerevisiae-Based Mutational Analysis of the bc_1 Complex Q_o Site Residue 279 To Study the Trade-Off between Atovaquone Resistance and Function

Zehua Song,[a] Jérôme Clain,[b,c] Bogdan I. Iorga,[d] Zhou Yi,[a] Nicholas Fisher,[e] Brigitte Meunier[a]

Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Gif-sur-Yvette, France[a]; UMR 216, Faculté de Pharmacie de Paris, Université Paris Descartes, Paris, France[b]; UMR 216, Institut de Recherche pour le Développement, Paris, France[c]; Institut de Chimie des Substances Naturelles, CNRS, UPR 2301, Labex LERMIT, Gif-sur-Yvette, France[d]; Plant Research Laboratory, Michigan State University, East Lansing, Michigan, USA[e]

The bc_1 complex is central to mitochondrial bioenergetics and the target of the antimalarial drug atovaquone that binds in the quinol oxidation (Q_o) site of the complex. Structural analysis has shown that the Q_o site residue Y279 (Y268 in *Plasmodium falciparum*) is key for atovaquone binding. Consequently, atovaquone resistance can be acquired by mutation of that residue. In addition to the probability of amino acid substitution, the level of atovaquone resistance and the loss of bc_1 complex activity that are associated with the novel amino acid would restrict the nature of resistance-driven mutations occurring on atovaquone exposure in native parasite populations. Using the yeast model, we characterized the effect of all the amino acid replacements resulting from a single nucleotide substitution at codon 279: Y279C, Y279D, Y279F, Y279H, Y279N, and Y279S (Y279C, D, F, H, N, and S). Two residue changes that required a double nucleotide substitution, Y279A and W, were added to the series. We found that mutations Y279A, C, and S conferred high atovaquone resistance but decreased the catalytic activity. Y279F had wild-type enzymatic activity and sensitivity to atovaquone, while the other substitutions caused a dramatic respiratory defect. The results obtained with the yeast model were examined in regard to atomic structure and compared to the reported data on the evolution of acquired atovaquone resistance in *P. falciparum*.

The mitochondrial respiratory chain complex III or bc_1 complex is central to mitochondrial bioenergetics and the target of antiprotozoals, such as atovaquone, and fungicidal drugs used to control human and plant pathogens. It is also currently the focus of intense research as an antimalarial target for further drug development (1–6). The bc_1 complex is a multimeric enzyme. Three subunits form the electron-transferring catalytic core and contain the redox-active groups, namely, cytochromes *b*, cytochrome c_1, and the "Rieske" iron-sulfur protein (ISP). Cytochrome *b* is mitochondrially encoded in all eukaryotes and contains the substrate ubiquinol/ubiquinone binding sites (i.e., the quinol oxidation [Q_o] and quinone reduction [Q_i] sites), which form the sites of competitive inhibition for antimicrobial agents. In the context of malaria, atovaquone (used in combination with proguanil, and marketed as Malarone) is a popular prophylactic drug and also shows high efficiency in the treatment of uncomplicated *Plasmodium falciparum* malaria.

Interestingly, atovaquone resistance frequently evolves through *de novo* cytochrome *b* mutations during antimalarial therapy (7–10). The resistance is caused by the mutation of residue Y279 (Y268 in *P. falciparum* numbering) in the atovaquone target site, the bc_1 complex Q_o site. Y279 is a highly conserved residue. It is crucial for stabilizing the bound atovaquone (11) and is postulated to play a key role in the binding and correct positioning of the ubiquinol in the Q_o site, facilitating a fast electron transfer to the [2Fe-2S] cluster of the ISP (12, 13).

In *P. falciparum*, two substitutions of Y279 are commonly associated with atovaquone resistance *in vivo*, namely, Y279S and Y279C (Y279S and C) (7, 9), whereas a third mutation, Y279N, is much less frequent (14). These three amino acid replacements correspond to single nucleotide substitutions at codon 279, suggesting that the evolutionary space is restricted to easily accessible amino acid replacements. Therefore, we hypothesized that the *in vivo* evolution of *P. falciparum* resistance to atovaquone results from a balance between various properties of residue 279—the probability of amino acid substitution, the level of atovaquone resistance, and the possible loss of bc_1 complex activity—that are associated with the novel amino acid.

Since *Plasmodium* parasites are not amenable to mitochondrial transformation, the effect of the different mutations (other than those observed in the wild) of Y279 on bc_1 complex activity and sensitivity to drugs cannot be assessed directly and an experimental model has to be used. Yeast (*Saccharomyces cerevisiae*) is amenable to mitochondrial transformation. Its cytochrome *b* shares a high level of sequence similarity with the parasite cytochrome *b*. Therefore, yeast provides a useful surrogate model to study the functional effect of mitochondrially encoded cytochrome *b* mutations. In addition, yeast can be grown in either respiratory or fermentative conditions, which facilitates the production of mutants with highly deleterious respiratory effects and their subse-

Received 26 March 2015 **Returned for modification** 7 April 2015
Accepted 19 April 2015

Accepted manuscript posted online 27 April 2015

Citation Song Z, Clain J, Iorga BI, Yi Z, Fisher N, Meunier B. 2015. *Saccharomyces cerevisiae*-based mutational analysis of the bc_1 complex Q_o site residue 279 to study the trade-off between atovaquone resistance and function. Antimicrob Agents Chemother 59:4053–4058. doi:10.1128/AAC.00710-15.

Address correspondence to Brigitte Meunier, meunier@cgm.cnrs-gif.fr.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00710-15.

doi:10.1128/AAC.00710-15

quent analysis. Following that strategy, Y279S and C yeast mutants have been previously produced and shown to combine high resistance to atovaquone and decreased activity (15, 16).

Here, in order to fully explore the mutational landscape of Y279 associated with atovaquone resistance, we studied, in the yeast model, the effect of all the amino acid replacements resulting from a single nucleotide substitution at codon 279, namely, Y279C, D, F, H, N, and S. To further explore the biochemical requirements at residue 279, we also analyzed two additional mutations, Y279A and W, which introduce a small hydrophobic and a bulky aromatic residue, respectively, although the amino acids replacement required a double nucleotide change of codon Y279.

We measured the effect of these mutations on the respiratory growth competence and on the bc_1 complex activity and sensitivity to inhibitors. Structural modeling was then used to examine the impact of the amino acid changes on the structure and function of the Q_o site.

MATERIALS AND METHODS

Materials and growth media. Equine cytochrome *c*, decylubiquinone, azoxystrobin, antimycin, atovaquone, superoxide dismutase (SOD), catalase, and NADH were obtained from Sigma-Aldrich. The following media were used for the growth of yeast: YPD (1% yeast extract, 2% peptone, and 3% glucose), YPGal (1% yeast extract, 2% peptone, and 2% galactose), YPeth (1% yeast extract, 2% peptone, and 2% ethanol), and YPG (1% yeast extract, 2% peptone, and 3% glycerol).

Yeast mitochondrial mutants. The mutants were generated by side-directed mutagenesis and mitochondrial transformation as described previously (17). They have identical nuclear and mitochondrial genomes, with the exception of the mutations introduced in the cytochrome *b* gene.

Measurement of NADH- and decylubiquinol-cytochrome *c* reductase activity. Yeast mitochondria were prepared as described previously (18). Briefly, yeast grown in YPGal medium were harvested at mid-log phase. Protoplasts were obtained by enzymatic digestion of the cell wall using zymolyase in an osmotic protection buffer. Mitochondria were then prepared by differential centrifugation following osmotic shock of the protoplasts. Mitochondrial samples were aliquoted and stored at −80°C. The concentration of the bc_1 complex in the mitochondrial samples was determined from dithionite-reduced optical spectra, using $\varepsilon = 28.5$ mM^{-1} cm^{-1} at 562 versus 575 nm. NADH- and decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 μM) at 550 nm versus 540 nm over a 1-min time course in 10 mM potassium phosphate (pH 7) and 1 mM KCN. Lauryl-maltoside (0.01% [wt/vol]) was added to the reaction buffer for the decylubiquinol-cytochrome *c* reduction assays. Mitochondria were added to obtain a final concentration of 5 to 30 nM bc_1 complex. Activity was initiated by the addition of decylubiquinol (final concentration, 5 to 20 μM), a synthetic analog of ubiquinol or by the addition of NADH (final concentration, 100 μM). Initial rates were measured. The measurements were repeated three to five times and averaged. Turnover numbers (TN) were determined as the cytochrome *c* reduction rate per bc_1 complex at 20 μM decylubiquinol or 100 μM NADH. K_m values were estimated from the plots of cytochrome *c* reduction rates *versus* decylubiquinol concentrations. The midpoint inhibition concentrations (IC_{50}) were determined by inhibitor titration.

Ligand docking and molecular modeling. Three-dimensional structure of decylubiquinol was generated using CORINA (Molecular Networks GmbH, Erlangen, Germany). The structure of the yeast bc_1 complex was downloaded from the Protein Data Bank (19) and used as receptor in the docking process. Water molecules, ligands, lipids, and hemes were removed, as well as all other chains except those corresponding to cytochrome *b* and ISP. Hydrogen atoms were added using HERMES, the graphical interface of GOLD (20). The docking of decylubiquinol was performed with GOLD using a binding site (the Q_o site) defined as a 15-Å radius sphere centered on the δ1-carbon atom of cytochrome *b* residue I147. GoldScore was used as a scoring function, and all other parameters had default values. *In silico* mutations Y279X were introduced using CHIMERA (21), which was also used for generating the molecular modeling images.

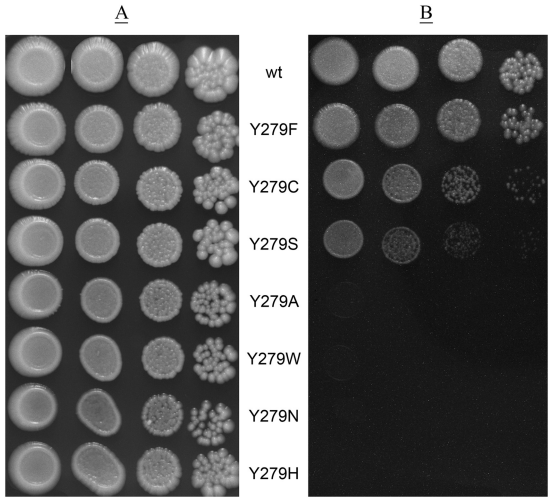

FIG 1 Respiratory growth competence of Y279 mutants. Serial dilutions in water of cells pregrown on glucose plates were spotted onto plates containing either glucose (YPD, fermentative medium) (A) or glycerol (YPG, respiratory medium) (B) and incubated for 3 days at 28°C.

RESULTS

Effect of substitution of Y279 in yeast bc_1 complex on the enzymatic activity and atovaquone sensitivity. Residue Y279 has a key role in binding atovaquone in the bc_1 complex Q_o site (11). Therefore, it is reasonable to expect that substitution of this residue may confer resistance to the drug. We studied the functional impact of all amino acid replacement resulting from a single nucleotide substitution of codon Y279 (UAU) in yeast cytochrome *b*, namely, Y279C (UGU), D (GAU), F (UUU), H (CAU), N (AAU), and S (UCU), because these are the most likely to occur *in vivo* during malaria therapy. Two amino acid replacements resulting from a double nucleotide substitution were also analyzed: Y279A and W. They introduce a small hydrophobic and a bulky aromatic residue, respectively.

The mutants were generated as described in reference 17.The respiratory growth competence (Fig. 1), the bc_1 complex activity measured as decylubiquinol- and NADH-cytochrome *c* reductase activities, and the sensitivity to Q_o and Q_i site inhibitors were then monitored (Table 1).

Whereas all the mutants grew, as well as the wild type (wt) in fermentative medium, in which the cell energy does not depend on a functional mitochondrial respiratory chain, large differences in respiratory growth rate were found among the mutant strains.

Mutations Y279D, H, N, and W caused a complete respiratory growth defect. Y279D was not studied further. Y279H, N, and W had lost ca. 95% of the control bc_1 complex activity, and the resistance to atovaquone could not be tested. As the introduction of charged or bulky residues at position 279 might destabilize the interaction with the ISP (which has been shown to be labile in some mutant strains [22]), we monitored the level of that subunit in the different mutants. It was previously shown that the replacement of the tyrosine by serine did not result in the loss of ISP (16).

TABLE 1 Effects of Y279 mutations on respiratory growth and bc_1 complex activity and sensitivity to inhibitors

Residue 279		Mean growth ± SD (%wt)[a]	Cytochrome *c* reduction (mean ± SD)[b]					
			Decylubiquinol cytochrome *c* reduction					NADH cytochrome *c* reduction turnover (%wt)
			Activity		IC_{50}/bc_1[c]			
Codon[d]	Amino acid[e]		Turnover (%wt)	K_m (μM)	Atovaquone	Azoxystrobin	Antimycin	
UAU	Y279	100 ± 5	100 ± 1	3.1 ± 0.32	4 ± 0.6	12 ± 1.2	0.3 ± 0.01	100 ± 5
U**U**U	Y279F	94 ± 4	98 ± 2	4.9 ± 0.40	2 ± 0.2	11 ± 0.7	0.3 ± 0.02	95 ± 2
U**G**U	**Y279C**	27 ± 2	24 ± 1	3.0 ± 0.15	180 ± 21	15 ± 0.7	0.3 ± 0.02	28 ± 1
U**C**U	**Y279S**	17 ± 3	24 ± 1	3.6 ± 0.15	193 ± 12	33 ± 2.1	0.2 ± 0.01	33 ± 1
GCU	Y279A	1 ± 0.5	18 ± 1	3.2 ± 0,12	154 ± 11	18 ± 2.2	0.4 ± 0.02	14 ± 0.2
GAU	Y279D	0	ND	ND	ND	ND	ND	ND
CAU	Y279H	0	6	ND	ND	ND	ND	ND
AAU	**Y279N**	0	6 ± 0.5	ND	ND	ND	ND	5 ± 1
U**GG**	Y279W	0	6 ± 0.4	ND	ND	ND	ND	6 ± 0.4

[a] Growth competence was monitored in YPEth medium. Culture started at an OD_{600} of 0.5. Samples were incubated with vigorous agitation for 3 days at 28°C. The OD_{600} values were then recorded. The values are presented as a percentage of the wild-type OD_{600} (%wt).

[b] The cytochrome *c* reduction activity was measured as described in Materials and Methods and is reported by the bc_1 complex concentration. The values are presented as a percentages of the wild-type activity (%wt). The wild-type decylubiquinol cytochrome *c* reduction rate was 53 ± 1.4 s^{-1} (mean ± the standard deviation); the wild-type NADH cytochrome *c* reduction rate was 106 ± 5.1 s^{-1} (mean ± the standard deviation). ND, not determined. All the measurements have been made three to five times (except for Y279H) and averaged.

[c] IC_{50}/bc_1, inhibitor concentration required to obtain 50% inhibition reported by the bc_1 complex concentration.

[d] Mutated bases are indicated by underlining.

[e] The amino acid at position 279 of cytochrome *b* (yeast numbering, which corresponds to position 268 in *P. falciparum*) found to be associated with clinical atovaquone resistance in *P. falciparum* is indicated in boldface.

By Western blotting, we confirmed these data and found that the level of ISP was not decreased in any of the mutants studied here (not shown). Thus, the mutations resulted in an assembled but inactive enzyme.

Y279C and S have been reported in *P. falciparum* and shown to cause atovaquone resistance (see, for instance, references 7 and 9). The same substitutions introduced in the yeast enzyme also conferred atovaquone resistance (~45-fold resistance compared to the wild type). The mutated complexes had 4-fold-lower decylubiquinol- and NADH-cytochrome *c* reductase activities, and the respiratory growth competence of the mutants was decreased. Y279A also combined atovaquone resistance with a decreased activity. This mutant, however, had a severe defect in respiratory growth due to the loss of more than 80% of the bc_1 complex activity. We have previously observed that there is a sharp fall in respiratory growth competence between 25 and 15% of bc_1 complex activity (unpublished data).

In contrast, mutation Y279F had minimal effect on growth, bc_1 complex activity or atovaquone sensitivity, except for a slightly higher K_m for decylubiquinol. The TN/K_m ratio (effectively a second-order rate constant for the interaction between substrate and enzyme) was slightly lower in the mutant, 13.6 and 17 for Y279F and wt, respectively, which indicates a mild decrease of the catalytic efficiency of the mutant enzyme.

As a control, we monitored the sensitivity of the wt and mutant enzymes to antimycin and azoxystrobin, two well-known bc_1 complex inhibitors that bind at different target sites. Y279F, C, S, and A, showed the same sensitivity as the wt to the Q_i site inhibitor antimycin. They also presented a very similar sensitivity to azoxystrobin that binds in the Q_o site but in position different to atovaquone, (i.e., closer to heme b_L [23]), except for Y279S, which showed a small, 2.7-fold resistance increase.

Finally, we tested the production of superoxide (SO) by the wt and mutant bc_1 complexes. Mutation of the Q_o site may cause catalytic cycle dysfunction that results in side reactions and SO production. We measured SO production by monitoring the SOD-sensitive rate of cytochrome *c* reduction. The difference between the reduction rate in the absence and the presence of added SOD indicates the contribution of the cytochrome *c* reduction by SO to the overall cytochrome *c* reductase activity. We compared the effect of the mutations Y279F, C, S, and A on SO production. The data are shown in Fig. 2.

The wt and mutant Y279F bc_1 complexes did not produce SO. No or little SO production was detected for Y279C. Around 11

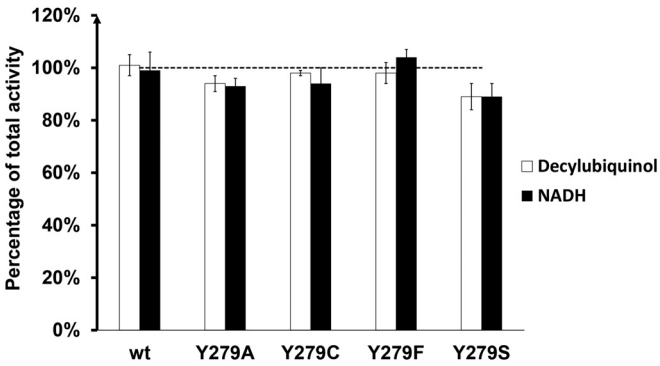

FIG 2 Superoxide production in the wild type and mutants. Cytochrome *c* reduction rates were recorded in the absence and presence of SOD and catalase, both at 225 U/ml, using 20 μM decylubiquinol or 100 μM NADH as the substrates. The assays were performed as described in Materials and Methods, with slight modifications made to optimize the SOD and catalase activities: decylubiquinol-cytochrome *c* reduction was assayed in 50 mM potassium phosphate buffer (pH 7) with 10 μM KCN, and NADH-cytochrome *c* reduction was monitored in 10 mM potassium phosphate buffer (pH 7.4) with 5 μM KCN. Each measurement was repeated at least three times and averaged. The decylubiquinol (white) and NADH (black) cytochrome *c* reduction rates in the presence of SOD and catalase are presented as percentages of the rates in the absence of SOD-catalase.

and 7% of the cytochrome *c* reduction activity of the enzyme could be attributed to SO in the Y279S and Y279A mutants, respectively. It was previously shown that Y279A, C, and S mutant enzymes exhibited a low SO production, a finding very similar to that observed here (24). Similar observations were reported using *Rhodobacter capsulatus* Y279 mutants (25).

The increase in SO production can be understood in terms of higher semiquinone occupancy at the Q_o site, increasing the equilibrium concentration of semiquinone for reaction with oxygen or an increased rate constant for the reaction with oxygen, perhaps by steric means (i.e., an increased probability of collision with oxygen). SO production observed in the Y279A and Y279S mutants in this study may reflect a prolonged occupancy of the semiquinone in the Q_o site and a slower semiquinone oxidation by the normal reaction partner, ferriheme b_L.

In summary, of the eight substitutions of Y279 studied here, Y279D, H, N, and W had a dramatic effect on the respiratory function. Y279A, C, and S significantly decreased the bc_1 complex activity, while Y279F resulted in a fully functional enzyme. The differential effect of the substitutions on the enzymatic activity could be explained by examining the Q_o site structure.

Residue 279 in the Q_o site structure. An atomic structure for ubiquinol bound within the Q_o site is not available. Nevertheless, the structure of the stigmatellin-inhibited enzyme is considered to provide a good model for the quinol binding at the Q_o site prior to the formation of the semiquinone anion and electron transfer to ferriheme b_L (PDB code 3CX5 [26]). Starting from that atomic structure, we also generated *in silico* models of the quinol-bound Q_o site (Fig. 3). These were used to examine the role of Y279 in quinol binding and to evaluate the impact of the substitutions of that residue.

The tyrosyl side chain of Y279 is likely to contribute to quinol binding in different ways. The residue makes a significant (65-Å^2) hydrophobic interaction with the chromone headgroup of stigmatellin and is likely to perform a similar function with the native quinol substrate.

The hydroxyl of Y279 could also form a hydrogen bond with the oxygen atom of ISP residue C180, which might act to stabilize the mobile domain of the ISP at the surface of the Q_o site, facilitating quinol oxidation. However, that hydrogen bond seems to have only a modest role since the replacement of tyrosine by phenylalanine has little effect on the bc_1 complex function as shown in Table 1, a finding which is in agreement with previous studies using bacterial enzymes (25, 27).

Y279 is also likely to contribute to the correct conformation of the *ef* loop comprising the highly conserved motif $PEWY_{271–274}$ and the *ef* helix 275-284, which would be required to maintain the correct structure of the catalytic cavity and to ensure an optimal binding of the substrate quinol (see reference 28) for a review). As presented in the model (Fig. 3a), Y279 is in the immediate vicinity of P271, which is part of the PEWY motif and conserved in almost all organisms (29). The proline ring and the tyrosine ring are in van der Waals contact. Their interaction might be important to maintain the optimal distance between I269 and ISP C180 (and thus the correct docking of the ISP) and between residue E272, key player in quinol oxidation (30), and the quinol.

Substitution of Y279 by nonaromatic residues would result in the loss of these stabilizing interactions and compromise the quinol binding and oxidation. This explains the loss of bc_1 complex activity resulting from the mutations Y279A, C, D, N, and S. The severity of the activity loss was found to increase in the following order: C > S > A > N/D. In bacteria, analysis of mutant bc_1 complexes showed that replacement of Y279 by valine or leucine had a moderate effect on the enzyme activity, that alanine and cysteine increased the severity, and that serine and glycine caused further activity loss. The most severely impaired mutant seemed to be Y279Q (25, 27). It thus appears that, in yeasts as in bacterial complexes, the severity of the effect is determined by both the size and the polarity of the side chain.

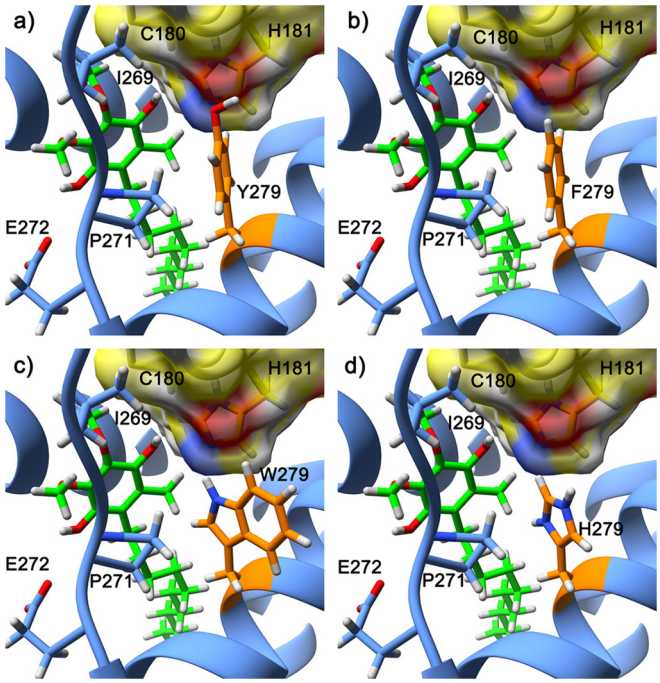

FIG 3 Location of residue 279 in the bc_1 complex Q_o site. (a) Wild-type amino acid Y279 in contact with P271 of the PEWY motif. (b to d) Substitution of Y279 by the aromatic residues F279 (b), W279 (c), and H279 (d). Cytochrome *b* is presented in blue. ISP is indicated in yellow with its surface colored by heteroatoms. Decylubiquinol is indicated in green. Residue 279 is indicated in orange. Oxygen atoms are indicated in red, nitrogen atoms are indicated in dark blue, and hydrogen atoms are indicated in light gray. The model has been constructed as described in Materials and Methods.

The effect of the substitution of Y279 by an aromatic residues was then examined. Based on the structural model (Fig. 3), it appeared that the aromatic residue phenylalanine (Fig. 3b) could fulfill the role of Y279, which explains that the substitution Y279F had no or only a very mild effect. In contrast, the introduction of the bulkier aromatic residue tryptophan (Fig. 3c) is likely to result in steric hindrance, impairing the structure of the Q_o site and thus its function. Substitution of the tyrosine by the charged residue histidine (Fig. 3d) would alter the environment of the quinol binding site. It is not tolerated in the yeast Q_o site because the mutation causes a dramatic loss of bc_1 complex activity.

The importance of an aromatic residue, tyrosine or phenylalanine, in position 279 is supported by the conservation of the residue among species. Using a large collection of available cytochrome *b* sequences (>9,500), we monitored the nature of the residue at the fifth position after the conserved PEWY motif, which corresponds to residue Y279 in yeast cytochrome *b* (see Table S1 in the supplemental material).

A tyrosine is conserved in the cytochrome *b* of almost all bacteria,

fungi, and protozoa and in all of the animals. In all of the *Plasmodium* sequences available in the GenBank database, the conservation of Y279 is respected besides sequences reported from clinical atovaquone-resistant cases, in which a serine was observed at this position. In archaea, tyrosine and phenylalanine are found in 61 and 39%, respectively, of the analyzed sequences. A tyrosine is also observed in 69% of plant cytochrome *b*, while in the remaining 31% of the available sequences, a histidine replaced the tyrosine at position 279. This is intriguing since, in yeast, the substitution of tyrosine by histidine results in an inactive enzyme. It may be postulated that the plant Q_o site has evolved for a better compatibility with a histidine and that the presence of this charged residue might be compensated for by other variations. Comparison of sequences of the amino acids in the vicinity of residue 279 did not reveal major differences, suggesting more distant or subtle effects. In all b_6f complex subunit IV sequences, a phenylalanine is found instead of a tyrosine. Subunit IV with cytochrome b_6 shows many structural and functional similarities to bc_1 complex cytochrome *b*. Comparison of the structures of the bc_1 and b_6f complex Q_o sites shows that the Y279 and the equivalent phenylalanine occupy very similar positions and orientations (not shown). Thus, the data indicate that, with the exception of some plants, the correct function of the Q_o site requires the aromatic residues tyrosine or phenylalanine.

DISCUSSION

The antimalarial drug atovaquone binds in the b_L distal region of the Q_o site, similarly to stigmatellin, and forms a strong hydrogen bond (2.8 Å) to the protonated imidazole ring of the ISP [2Fe-2S] cluster H181 in the recently published yeast atomic structure (PDB code 4PD4 [11]). The tyrosyl side chain of residue Y279 (Y268 in *P. falciparum*) makes a significant, stabilizing aromatic contact with the hydroxynaphthoquinone moiety of Q_o-bound atovaquone. That aromatic interaction can also be formed by a phenylalanine. Other substitutions resulting in the loss of that key stabilizing interaction would thus cause atovaquone resistance, which is associated with atovaquone-proguanil treatment failure in malaria.

Using the yeast model, we studied eight substitutions of Y279. Six of these amino acid replacements (Y279C, D, F, H, N, and S) resulted from a single nucleotide substitution of codon 279, while two (Y279A and W) required a double nucleotide substitution. The consequences of the mutations on the respiratory growth competence and on the bc_1 complex activity and sensitivity to atovaquone were investigated. The structural basis of the effects of the mutations was then examined.

The substitution Y279F had no or little effect on bc_1 complex activity and sensitivity to atovaquone. This was expected from the examination of the Q_o structures that indicates that tyrosine and phenylalanine could be swapped without deleterious effect and without loss of the stabilizing interaction with atovaquone. Four mutations (Y279D, H, N, and W) lead to a dramatic loss of respiratory function. The atovaquone sensitivity could not be tested. Three substitutions (Y279A, C, and S) resulted in atovaquone resistance combined with decreased activity.

Acquisition of atovaquone resistance in malaria parasites needs three requirements to be fulfilled: a high probability of the amino acid replacement, a high level of atovaquone resistance, and a limited loss of fitness associated with the resistance mutation. Based on the yeast analysis, three mutations of Y279 fulfill the atovaquone resistance and fitness criteria: Y279C, S, and A. However, whereas Y279C and S amino acid replacements result from a single nucleotide substitution, Y279A requires a double nucleotide substitution, which makes it unlikely to occur on the short time scale of a malaria treatment. This is fully consistent with Y279A not being found in any of the atovaquone-resistant parasites reported to date and with Y279C and S being the two major mutations found in atovaquone-resistant parasites from atovaquone-proguanil treatment failures (7, 9).

A third mutation, Y279N, has been reported in association with clinical atovaquone resistance (7, 9, 14). Based on the data obtained with the yeast model, that mutation would not be expected to be tolerated since it causes a severe loss of function. However, reported studies indicate that, in the parasite as in yeast, Y279N has a more deleterious impact than Y279C and S. First, analysis of rodent malaria parasites showed reduced *in vivo* growth rate and bc_1 complex activity for Y279N mutant strains compared to Y279C (31, 32). Also, a heavier fitness penalty is suggested by the rate at which the atovaquone resistance mutations emerged and were selected during malaria therapy with Y279S, C, and N representing roughly 54, 42, and 4% of mutant occurrence, respectively ($n = 11$, 14, and 1 for Y279C, S, and N, respectively [9]; see also Table S2 in the supplemental material). Minor structural differences in the Q_o site structure between the parasite and yeast enzymes might result in a more stringent effect of Y279N in the yeast enzyme.

In parasites, as in yeasts, the acquisition of atovaquone resistance is associated with the loss of bc_1 complex activity and decreased fitness (33). Resistance mutations would be expected to be counterselected in the absence of drug pressure and thus not detected in the parasite cytochrome *b* gene of field isolates. Atovaquone-proguanil being poorly used in areas of endemicity because of its high cost, it is therefore intriguing that the mutations Y279C, S, and N have been reported in field isolates from Kenya at rather high frequency (34). However, since the level of drug use was not documented, interpretations of these data should be taken with caution. It might be hypothesized that other polymorphisms in the Q_o domain, in cytochrome *b* or ISP, partly compensate for the loss of the tyrosine. The genetic compensation would result in a more active enzyme and a reduced fitness cost. In yeast, we found that changes in the ISP hinge region could partially restore the respiratory function severely altered by the Q_o site mutations G291D, S152P, and A144F (22, 35).

The bc_1 complex is an attractive target for antimicrobial drugs. Atovaquone is currently the sole drug in clinical use targeting that enzyme. We analyzed in the yeast model the trade-off between atovaquone resistance and bc_1 complex activity that associates with mutations or Y279. We showed that cysteine and serine are optimal atovaquone-resistant amino acid at position. These results parallel the *in vivo* evolution of atovaquone resistance during malaria therapy and support the yeast model as a useful surrogate to study antimalarial drug resistance.

ACKNOWLEDGMENT

Z.S. holds a China Scholarship Council Studentship. This study was supported by funding from the Fondation pour la Recherche Médicale.

REFERENCES

1. **Nam T-G, McNamara CW, Bopp S, Dharia NV, Meister S, Bonamy GMC, Plouffe DM, Kato N, McCormack S, Bursulaya B, Ke H, Vaidya AB, Schultz PG, Winzeler EA.** 2011. A chemical genomic analysis of decoquinate, a *Plasmodium falciparum* cytochrome *b* inhibitor. ACS Chem Biol **6**:1214–1222. http://dx.doi.org/10.1021/cb200105d.
2. **Biagini GA, Fisher N, Shone AE, Mubaraki MA, Srivastava A, Hill A,**

Antoine T, Warman AJ, Davies J, Pidathala C, Amewu RK, Leung SC, Sharma R, Gibbons P, Hong DW, Pacorel B, Lawrenson AS, Charoensutthivarakul S, Taylor L, Berger O, Mbekeani A, Stocks PA, Nixon GL, Chadwick J, Hemingway J, Delves MJ, Sinden RE, Zeeman A-M, Kocken CHM, Berry NG, O'Neill PM, Ward SA. 2012. Generation of quinolone antimalarials targeting the *Plasmodium falciparum* mitochondrial respiratory chain for the treatment and prophylaxis of malaria. Proc Natl Acad Sci U S A **109:**8298–8303. http://dx.doi.org/10.1073/pnas.1205651109.

3. **Nilsen A, LaCrue AN, White KL, Forquer IP, Cross RM, Marfurt J, Mather MW, Delves MJ, Shackleford DM, Saenz FE, Morrisey JM, Steuten J, Mutka T, Li Y, Wirjanata G, Ryan E, Duffy S, Kelly JX, Sebayang BF, Zeeman A-M, Noviyanti R, Sinden RE, Kocken CHM, Price RN, Avery VM, Angulo-Barturen I, Jiménez-Díaz MB, Ferrer S, Herreros E, Sanz LM, Gamo F-J, Bathurst I, Burrows JN, Siegl P, Guy RK, Winter RW, Vaidya AB, Charman SA, Kyle DE, Manetsch R, Riscoe MK.** 2013. Quinolone-3-diarylethers: a new class of antimalarial drug. Sci Transl Med **5:**177ra37. http://dx.doi.org/10.1126/scitranslmed.3005029.
4. **Dong CK, Urgaonkar S, Cortese JF, Gamo F-J, Garcia-Bustos JF, Lafuente MJ, Patel V, Ross L, Coleman BI, Derbyshire ER, Clish CB, Serrano AE, Cromwell M, Barker RH, Dvorin JD, Duraisingh MT, Wirth DF, Clardy J, Mazitschek R.** 2011. Identification and validation of tetracyclic benzothiazepines as *Plasmodium falciparum* cytochrome bc_1 inhibitors. Chem Biol **18:**1602–1610. http://dx.doi.org/10.1016/j.chembiol.2011.09.016.
5. **Capper MJ, O'Neill PM, Fisher N, Strange RW, Moss D, Ward SA, Berry NG, Lawrenson AS, Hasnain SS, Biagini GA, Antonyuk SV.** 2015. Antimalarial 4(1*H*)-pyridones bind to the Q_i site of cytochrome bc_1. Proc Natl Acad Sci U S A **112:**755–760. http://dx.doi.org/10.1073/pnas.1416611112.
6. **Stickles AM, Justino de Almeida M, Morrisey JM, Sheridan KA, Forquer IP, Nilsen A, Winter RW, Burrows JN, Fidock DA, Vaidya AB, Riscoe MK.** 2015. Subtle changes in endochin-like quinolone (ELQ) structure alter site of inhibition within the cytochrome bc_1 complex of *Plasmodium falciparum*. Antimicrob Agents Chemother **59:**1977–1982. http://dx.doi.org/10.1128/AAC.04149-14.
7. **Musset L, Bouchaud O, Matheron S, Massias L, Le Bras J.** 2006. Clinical atovaquone-proguanil resistance of *Plasmodium falciparum* associated with cytochrome *b* codon 268 mutations. Microbes Infect **8:**2599–2604. http://dx.doi.org/10.1016/j.micinf.2006.07.011.
8. **Musset L, Le Bras J, Clain J.** 2007. Parallel evolution of adaptive mutations in *Plasmodium falciparum* mitochondrial DNA during atovaquone-proguanil treatment. Mol Biol Evol **24:**1582–1585. http://dx.doi.org/10.1093/molbev/msm087.
9. **Cottrell G, Musset L, Hubert V, Le Bras J, Clain J.** 2014. Emergence of resistance to atovaquone-proguanil in malaria parasites: insights from computational modeling and clinical case reports. Antimicrob Agents Chemother **58:**4504–4514. http://dx.doi.org/10.1128/AAC.02550-13.
10. **Schwartz E, Bujanover S, Kain KC.** 2003. Genetic confirmation of atovaquone-proguanil-resistant *Plasmodium falciparum* malaria acquired by a nonimmune traveler to East Africa. Clin Infect Dis **37:**450–451. http://dx.doi.org/10.1086/375599.
11. **Birth D, Kao W-C, Hunte C.** 2014. Structural analysis of atovaquone-inhibited cytochrome bc_1 complex reveals the molecular basis of antimalarial drug action. Nat Commun **5:**4029. http://dx.doi.org/10.1038/ncomms5029.
12. **Palsdottir H, Lojero CG, Trumpower BL, Hunte C.** 2003. Structure of the yeast cytochrome bc_1 complex with a hydroxyquinone anion Q_o site inhibitor bound. J Biol Chem **278:**31303–31311. http://dx.doi.org/10.1074/jbc.M302195200.
13. **Barragan AM, Crofts AR, Schulten K, Solov IA.** 2014. Identification of ubiquinol binding motifs at the Q_o site of the cytochrome bc_1 complex. J Phys Chem B **119:**433–447. http://dx.doi.org/10.1021/jp510022w.
14. **Sutherland CJ, Laundy M, Price N, Burke M, Fivelman QL, Pasvol G, Klein JL, Chiodini PL.** 2008. Mutations in the *Plasmodium falciparum* cytochrome *b* gene are associated with delayed parasite recrudescence in malaria patients treated with atovaquone-proguanil. Malar J **7:**240. http://dx.doi.org/10.1186/1475-2875-7-240.
15. **Fisher N, Meunier B.** 2008. Molecular basis of resistance to cytochrome bc_1 inhibitors. FEMS Yeast Res **8:**183–192. http://dx.doi.org/10.1111/j.1567-1364.2007.00328.x.
16. **Kessl JJ, Ha KH, Merritt AK, Lange BB, Hill P, Meunier B, Meshnick SR, Trumpower BL.** 2005. Cytochrome *b* mutations that modify the ubiquinol-binding pocket of the cytochrome bc_1 complex and confer antimalarial drug resistance in *Saccharomyces cerevisiae*. J Biol Chem **280:**17142–17148. http://dx.doi.org/10.1074/jbc.M500388200.
17. **Hill P, Kessl J, Fisher N, Meshnick S, Trumpower BL, Meunier B.** 2003. Recapitulation in *Saccharomyces cerevisiae* of cytochrome *b* mutations conferring resistance to atovaquone in *Pneumocystis jiroveci* Antimicrob Agents Chemother **47:**2725–2731.
18. **Lemaire C, Dujardin G.** 2008. Preparation of respiratory chain complexes from *Saccharomyces cerevisiae* wild-type and mutant mitochondria: activity measurement and subunit composition analysis. Methods Mol Biol **432:**65–81. http://dx.doi.org/10.1007/978-1-59745-028-7_5.
19. **Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE.** 2000. The Protein Data Bank. Nucleic Acids Res **28:**235–242. http://dx.doi.org/10.1093/nar/28.1.235.
20. **Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD.** 2003. Improved protein-ligand docking using GOLD. Proteins Struct Funct Genet **52:**609–623. http://dx.doi.org/10.1002/prot.10465.
21. **Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE.** 2004. UCSF Chimera: a visualization system for exploratory research and analysis. J Comput Chem **25:**1605–1612. http://dx.doi.org/10.1002/jcc.20084.
22. **Fisher N, Castleden CK, Bourges I, Brasseur G, Dujardin G, Meunier B.** 2004. Human disease-related mutations in cytochrome *b* studied in yeast. J Biol Chem **279:**12951–12958. http://dx.doi.org/10.1074/jbc.M313866200.
23. **Esser L, Quinn B, Li Y-F, Zhang M, Elberry M, Yu L, Yu C-A, Xia D.** 2004. Crystallographic studies of quinol oxidation site inhibitors: a modified classification of inhibitors for the cytochrome bc_1 complex. J Mol Biol **341:**281–302. http://dx.doi.org/10.1016/j.jmb.2004.05.065.
24. **Wenz T, Covian R, Hellwig P, Macmillan F, Meunier B, Trumpower BL, Hunte C.** 2007. Mutational analysis of cytochrome *b* at the ubiquinol oxidation site of yeast complex III. J Biol Chem **282:**3977–3988.
25. **Lee D-W, Selamoglu N, Lanciano P, Cooley JW, Forquer I, Kramer DM, Daldal F.** 2011. Loss of a conserved tyrosine residue of cytochrome *b* induces reactive oxygen species production by cytochrome bc_1. J Biol Chem **286:**18139–18148. http://dx.doi.org/10.1074/jbc.M110.214460.
26. **Solmaz SRN, Hunte C.** 2008. Structure of complex III with bound cytochrome *c* in reduced state and definition of a minimal core interface for electron transfer. J Biol Chem **283:**17542–17549. http://dx.doi.org/10.1074/jbc.M710126200.
27. **Crofts AR, Guergova-Kuras M, Kuras R, Ugulava N, Li J, Hong S.** 2000. Proton-coupled electron transfer at the Q_o site: what type of mechanism can account for the high activation barrier? Biochim Biophys Acta **1459:**456–466. http://dx.doi.org/10.1016/S0005-2728(00)00184-5.
28. **Berry EA, Huang L-S.** 2011. Conformationally linked interaction in the cytochrome bc_1 complex between inhibitors of the Q_o site and the Rieske iron-sulfur protein. Biochim Biophys Acta **1807:**1349–1363. http://dx.doi.org/10.1016/j.bbabio.2011.04.005.
29. **Kao W-C, Hunte C.** 2014. The molecular evolution of the Q_o motif. Genome Biol Evol **6:**1894–1910. http://dx.doi.org/10.1093/gbe/evu147.
30. **Crofts AR, Hong S, Wilson C, Burton R, Victoria D, Harrison C, Schulten K.** 2013. The mechanism of ubihydroquinone oxidation at the Q_o site of the cytochrome bc_1 complex. Biochim Biophys Acta **1827:**1362–1377. http://dx.doi.org/10.1016/j.bbabio.2013.01.009.
31. **Siregar JE, Kurisu G, Kobayashi T, Matsuzaki M, Sakamoto K, Mi-Ichi F, Watanabe Y-I, Hirai M, Matsuoka H, Syafruddin D, Marzuki S, Kita K.** 2015. Direct evidence for the atovaquone action on the *Plasmodium* cytochrome bc_1 complex. Parasitol Int **64:**295–300. http://dx.doi.org/10.1016/j.parint.2015.03.005, http://dx.doi.org/10.1016/j.parint.2014.09.011.
32. **Siregar JE, Syafruddin D, Matsuoka H, Kita K, Marzuki S.** 2008. Mutation underlying resistance of *Plasmodium berghei* to atovaquone in the quinone binding domain 2 (Q_o 2) of the cytochrome *b* gene. Parasitol Int **57:**229–232. http://dx.doi.org/10.1016/j.parint.2007.12.002.
33. **Fisher N, Abd Majid R, Antoine T, Al-Helal M, Warman AJ, Johnson DJ, Lawrenson AS, Ranson H, O'Neill PM, Ward SA, Biagini GA.** 2012. Cytochrome *b* mutation Y268S conferring atovaquone resistance phenotype in malaria parasite results in reduced parasite bc_1 catalytic turnover and protein expression. J Biol Chem **287:**9731–9741. http://dx.doi.org/10.1074/jbc.M111.324319.
34. **Ingasia LA, Akala HM, Imbuga MO, Opot BH, Eyase FL, Johnson JD, Bulimo WD, Kamau E.** 2015. Molecular characterization of the cytochrome *b* gene and *in vitro* atovaquone susceptibility of *Plasmodium falciparum* isolates from Kenya. Antimicrob Agents Chemother **59:**1818–1821. http://dx.doi.org/10.1128/AAC.03956-14.
35. **Brasseur G, Lemesle-Meunier D, Reinaud F, Meunier B.** 2004. Q_o site deficiency can be compensated by extragenic mutations in the hinge region of the iron-sulfur protein in the bc_1 complex of *Saccharomyces cerevisiae*. J Biol Chem **279:**24203–24211. http://dx.doi.org/10.1074/jbc.M311576200.